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Antioxidant system parameters in boar spermatozoa of different morphology and motility

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ABSTRACT

The proportion of different functional and structural spermatozoa subpopulations and the level of antioxidative systems in semen are associated with the ability for fertilization and storing semen. The aim of this study was to determine the antioxidant system parameters in boar spermatozoa fractions separated in an iodixanol solution. Semen samples of 27 boars, aged between 1.5 and 3 years, were collected in early autumn. Semen samples were centrifuged in a discontinuous iodixanol density gradient solution, and three semen fractions were obtained: spermatozoa with high motility, spermatozoa with very low motility, and abnormal spermatozoa. The values of total antioxidant status (TAS), superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px) in each semen fraction were determined spectrophotometrically. In the abnormal spermatozoa fraction, the values of the TAS and the GSH-Px were significantly lower ($P<0.001$), and the SOD was significantly higher than in fractions with high or very low spermatozoa motility ($P<0.001$ and $P<0.05$, respectively). The values of TAS, SOD and GSH-Px were significantly higher in spermatozoa with very low motility than in spermatozoa with high motility ($P<0.001$). Higher values of SOD obtained in abnormal spermatozoa and spermatozoa with very low motility were associated with impaired spermatozoa function, motility and morphology. Significantly lower values of TAS and GSH-Px ($P<0.001$) in abnormal spermatozoa indicate a decrease in antioxidative protection. The higher values of TAS and GSH-Px obtained in spermatozoa with very low motility than in spermatozoa with high motility could indicate enhanced antioxidative protection due to the increased production of the reactive oxygen species. Determination of antioxidant parameters in different semen fractions may help to explain the potential causes of infertility in boars.

Key words: antioxidant, iodixanol, semen, boar

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Introduction

Sperm cellular integrity may be exposed to many harmful effects, but oxidative stress may cause the most damaging effect and result in male infertility, which is due to an increased production of reactive oxygen species (ROS) and/or disturbance of the balance between oxidants and antioxidants (ROCA et al., 2005). Boar spermatozoa, in their cell membrane phospholipids, contain large amounts of polyunsaturated fatty acids, and have a relatively low antioxidant capacity, which makes them very susceptible to oxidative damage (KOWALOWKA et al., 2008). The cell oxidative status is maintained by enzymatic and non-enzymatic antioxidants, to provide the optimal amounts of ROS necessary for the physiological functions of the sperm (FORD, 2004; SANOCKA and KURPISZ, 2004). However, antioxidant enzymes have an important function in protecting sperm from the ROS generated within the sperm cell. The most effective enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px), among which SOD is the most significant antioxidant (OGBUEWU et al., 2010; VALLORANI et al., 2010). SOD activity increases with the elevation of oxygen concentration *in vivo*, and its biological function is manifested in the transformation of superoxide radicals (O_2^-) to less toxic hydrogen peroxide (H_2O_2) (FRIDOVICH, 1995; HALLIWELL and GUTTERIDGE, 2007), which is neutralized by GSH-Px and CAT. The GSH-Px catalyses the reduction of hydrogen peroxide and/or organic hydro peroxides to water and the corresponding alcohols, using reduced glutathione (CHABORY et al., 2010). GSH-Px is the most significant antioxidative enzyme in protection against low levels of oxidative stress, while CAT is important for the direct removal of H_2O_2 during high levels of oxidative stress (VALKO et al., 2006). The presence and concentrations of antioxidants vary in spermatozoa among animal species (STRZEZEK et al., 2009).

Boar semen is composed of heterogeneous subpopulations of spermatozoa that are distinguished by their functional and structural features, and the proportion of each subpopulation is associated with the ability for fertilization and storing semen (MATÁS et al., 2011; GOVINDASAMY et al., 2016).

In particular, damaged and dead spermatozoa produce significant levels of ROS that may be toxic for them and for the healthy spermatozoa in the semen (SIKKA, 2004; O'FLAHERTY, 2014). Excessive amounts of ROS may disrupt the structure of the cell membrane and render spermatozoa dysfunctional. In addition, the ROS may cause damage to membrane lipids and proteins, and cause damage to the integrity of the DNA in the spermatozoa nucleus, which is associated with reduced sperm motility and apoptosis, while reducing the spermatozoa number and semen quality (AGARWAL et al., 2003; ZAKOŠEK PIPAN et al., 2014). Immature spermatozoa in excess cytoplasm contain enzymes that stimulate the additional generation of ROS, which can result in infertility, if the ejaculate contains a significant number of such spermatozoa (DE LAMIRANDE

and GAGNON, 1995; AITKEN and SAWYER, 2003). Therefore, in normal physiological conditions for normal sperm function and survival it is necessary to maintain the delicate balance between the formation and removal of free radicals (SHINDE et al., 2012).

Oxidative stress and antioxidant protection are scientific challenges that have been intensively investigated in the past several years in the field of biomedicine. Until now, in veterinary medicine the antioxidant parameters have not been determined in spermatozoa of different morphology and motility, but these values and interrelationships could help to explain the causes of male infertility. Therefore, the aim of this study was to separate boar spermatozoa of normal morphology and high motility from that of very low motility, and from abnormal spermatozoa in an iodixanol density solution, and to determine the presence and quantities of antioxidative system parameters tested in the separated semen fractions.

Materials and methods

Animals. The study was performed on 27 boars of different breeds (German Landrace, Swedish Landrace, Pietrain, Large White) and Pig Improvement Company hybrid (PIC hybrid), aged between 1.5 and 3 years, which were randomly selected in the Centre for Reproduction of Croatian Livestock, Work Unit for production in Križevci, Croatia. During the experimental period the animals were kept in separate compartments of 12 m^2 in size, and each compartment was fenced with a covered outlet in which zoo-hygienic measures were regularly conducted. The animals were fed once a day, before mounting with 3 kg of commercial feed produced by Poljoprivredna zadruga Virje, Virje, Croatia (Table 1). Water was given to the boars *ad libitum* by automatic watering.

Table 1. The composition of feed for the boars

Crude protein (%)	16
Crude fat (%)	16
Crude fiber (%)	7
Phosphorus (%)	0.6
Calcium (%)	1
Sodium (%)	0.25
Cink (mg/kg)	50
Vitamin A (IJ/kg)	5 000
Vitamin D3 (IJ/kg)	625

Semen sampling and processing. Semen samples were collected during routine exploitation of boars, conducted twice a week, in order to obtain semen for artificial insemination. Semen samples from selected boars were taken once in the early autumn

(late September and early October). Before each ejaculation, boars were stimulated by being taken to a separate room that contained a phantom sow, and the semen was obtained by manual fixation of the penis after it mounted the phantom. The last two semen fractions were collected in graduated sperm collection containers, having been strained through three layers of sterile gauze to separate the bulbourethral gland secretions. Following semen sampling, semen purity and macroscopic evaluation (volume, colour, consistency and odour) was performed. After this evaluation, which took a few seconds, strained ejaculates were placed in a water bath heated to 37 °C, in which they remained during the microscopic evaluation and determination of semen density. By microscopic evaluation of fresh semen the spermatozoa motility and morphologic spermatozoa features were estimated by visualization, using a phase-contrast system microscope with a warm stage (Olympus BX50F, Tokyo, Japan). The concentration of the spermatozoa was objectively determined using a Photometer SDM 5 (MiniTüb, Landshut, Germany). Semen samples which satisfied the criteria of macroscopic and microscopic evaluations and the concentration of spermatozoa (150-250×10^6 spermatozoa/mL) were taken for further processing.

The separation of spermatozoa in iodixanol density gradient. Each fresh ejaculate sample was prepared for centrifugation in discontinuous iodixanol density gradient (OptiPrep™, Axis, Norway), according to the manufacturer's instructions. OptiPrep™ is a 60% (w/v) solution of iodixanol in water with 1.32 g/mL density. Equal volumes of semen and 60% iodixanol solution were mixed to obtain a density of 1.170 g/mL. The prepared semen layer was overlaid with two iodixanol solutions of different density, which were prepared from iodixanol and Hanks Buffered Salt Solution (HANKS, 1975). The first solution was of 1.154 g/mL density, and the second of 1.119 g/mL density. The density gradient was centrifuged at 1500 g for 20 min at room temperature (about 20 °C), according to the manufacturer's instructions. After centrifugation, three semen fractions were obtained. In the fraction between the layers of 1.119 g/mL and 1.154 g/mL iodixanol density solutions, spermatozoa were isolated of normal morphology and high motility (>90%). In the precipitate were the very low motility (<20%) spermatozoa, while in the top gradient, abnormal spermatozoa were accumulated (deformed spermatozoa, spermatozoa with cytoplasmic droplet, detached spermatozoa heads and tails). All three semen fractions were stored at -80 °C until used for analyses.

Determination of antioxidant system parameters. In the separated spermatozoa samples, antioxidant status was monitored by determining the total antioxidative status (TAS), GSH-Px and SOD. Parameters of antioxidant protection in the spermatozoa samples, isolated by density-gradient were determined spectrophotemetrically using an automatic biochemistry analyser, Olympus AU 400 (Olympus, Tokyo, Japan) with commercial Randox kits (Randox). The concentration of the TAS was determined by

a commercial Total Antioxidant Status kit, the activity of GSH-Px by a commercial Ransel kit, and the activity of SOD by a commercial Ransod kit. The resulting values are expressed per spermatozoa count in the fractions.

Statistical analysis. The results were statistically processed using the SAS software package (Statistical Analysis Software) 9.1.3. Service Pack 4 (2002 - 2003 by SAS Institute Inc., SAS Institute Inc. URL: http://support.sas.com/onlinedoc/913/docMainpage.jsp., 29. September 2012.Cary, USA). The transformation of data was made when the assumption was that the normal distribution of the analysed dependent variables was disturbed, and with the heterogeneity of variances of different groups. The general linear model (GLM PROC) was used to analyse the variables between the different factions of spermatozoa. Results are expressed as the mean and 95% confidence interval (95% Cl), and were calculated using the least squares (LSM - least squares means) using LSMEANS commands and options PDIFF and CL. The Tukey-Kramer's method of multiple comparison was used to compare antioxidative system parameter means, at the level of statistical significance of $P<0.05$. After statistical analysis, the results, if the data were transformed, were returned by reverse transformation to the original values (mean and 95% Cl) and as such are shown in the text or graph.

Results

The results of the concentrations of the TAS in the boar spermatozoa of different morphological features and motility are presented in Fig. 1.

The statistical analysis showed significant differences in the concentration of TAS between all semen fractions obtained. Thus, the concentration of the TAS in spermatozoa with very low motility was significantly higher than that in spermatozoa samples with high motility and abnormal spermatozoa ($P<0.001$). At the same time, the concentration of the TAS in spermatozoa samples with high motility was significantly higher than that in abnormal spermatozoa ($P<0.001$).

The results of the activity of the GSH-Px in boar spermatozoa of different morphological features and motility are presented in Fig. 2.

The statistical analysis showed significant differences in GSH-Px activity between all the obtained semen fractions, and the GSH-Px activity in spermatozoa with very low motility was significantly higher than that in spermatozoa samples with high motility, and in abnormal spermatozoa ($P<0.001$). At the same time, GSH-Px activity in spermatozoa samples with high motility was significantly higher than that in abnormal spermatozoa ($P<0.001$).

The results of the activity of the SOD in boar spermatozoa of different morphological features and motility are presented in Fig. 3.

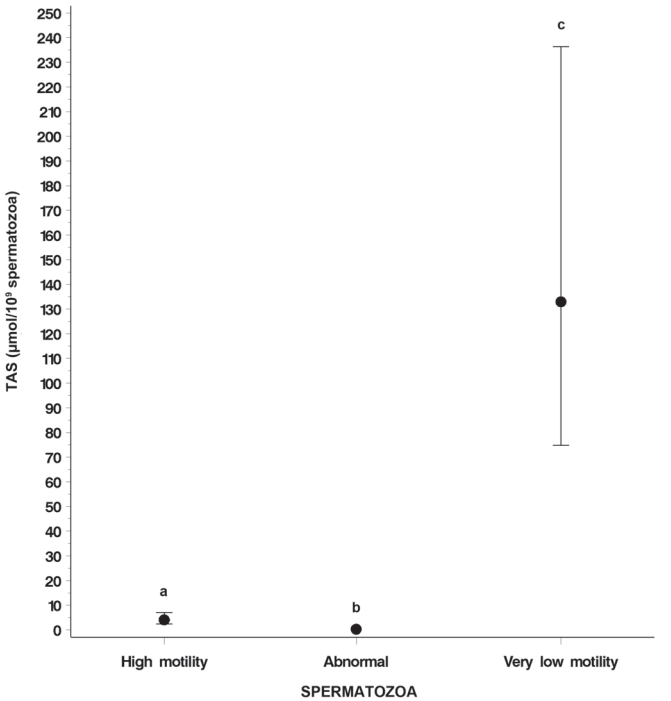

Fig. 1. The concentration of TAS in the boar spermatozoa of different morphological features and motility. The results are expressed as mean and 95% confidence interval of the mean (95% CI). Differently printed letters indicate a statistical significance between samples at the level of P<0.001.

By comparing the values of the SOD in different spermatozoa samples, statistically significant differences were found. The abnormal spermatozoa had significantly higher SOD activity in relation to the spermatozoa samples with high motility (P<0.01) and spermatozoa with very low motility (P<0.05). At the same time, spermatozoa samples with high motility had significantly lower SOD activity compared to the spermatozoa with very low motility (P<0.001).

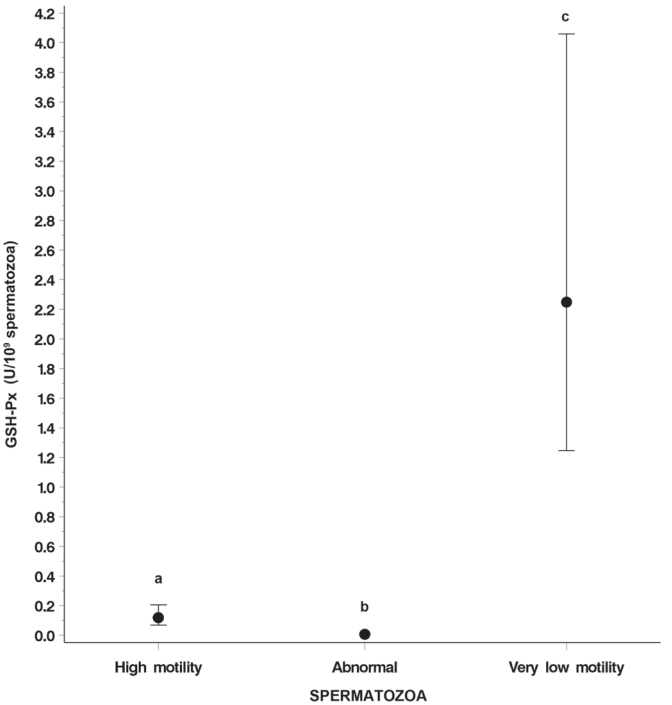

Fig. 2. The activity of GSH-Px in boar spermatozoa of different morphological features and motility. The results are expressed as mean and 95% confidence interval of the mean (95% CI). Different printed letters indicate a statistical significance between samples at the level of $P<0.001$.

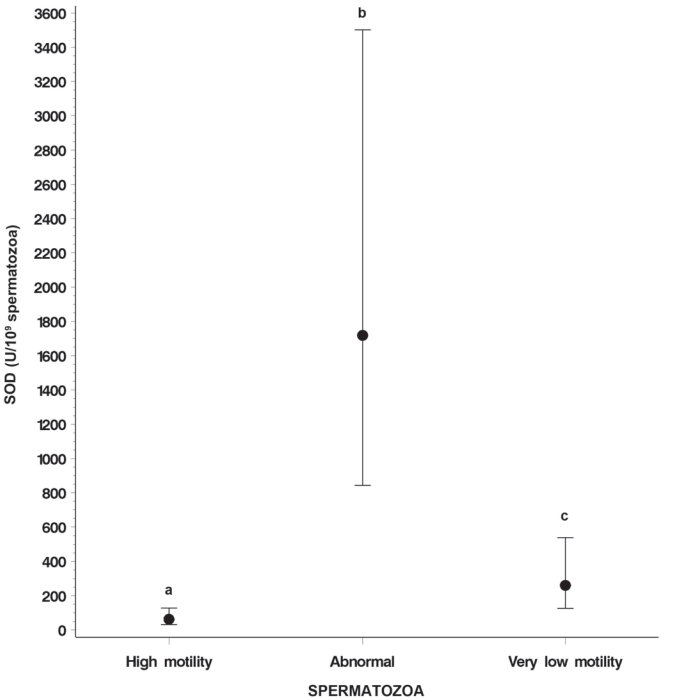

Fig. 3. The activity of the SOD in boar spermatozoa of different morphological features and motility. The results are expressed as mean and 95% confidence interval of the mean (95%CI). Different printed letters indicate a statistical significance between samples at the level of $P<0.05$.

Discussion

The results of this study indicate the several times higher activity of SOD compared to GSH-Px activity in boar spermatozoa, which is in accordance with the results of STRZEZEK et al. (2009). Similar results were obtained by KOZIOROWSKA-GILUN et al. (2011), who found, in boar spermatozoa originating from the tail of the epididymis, high activity of SOD and low activity of phospholipid hydroperoxide glutathione peroxidase (PHGSH-Px). The enzymatic antioxidant system in the boar spermatozoa is very deficient in contrast to other animal species, leading to high activity of SOD and low GSH-Px activity.

For example, stallion spermatozoa are exceptionally well protected from damage caused by ROS in comparison with boar spermatozoa, which have a limited antioxidative system (STRZEZEK et al., 1999; KOWALOWKA et al., 2008; STRZEZEK et al., 2009). The average activity of SOD in boar spermatozoa, which CASTELLANO et al. (2010) found, was 10.85 - 11.13 U/10^9 of spermatozoa, whereas the value found for SOD in this study

was higher. KADIRVEL et al. (2014) and LECEWICZ et al. (2015) found several times higher GSH-Px activity in the bull spermatozoa, in relation to the GSH-Px activity found in boar spermatozoa in our study. Similar findings were reported by STRZEZEK et al. (1999), KOWALOWKA et al. (2008) and STRZEZEK et al. (2009). The reduced antioxidant defences of boar spermatozoa in relation to other animal species could be one of the causes of the poor capability for freezing boar semen. Namely, the semen freezing process generates an additional amount of ROS, which further reduces the antioxidant protection of boar spermatozoa (AWDA et al., 2009; KUMARESAN et al., 2009).

The SOD activity found in the current study was significantly higher in the fraction of abnormal spermatozoa as compared to spermatozoa with high and very low motility, while spermatozoa with very low motility had significantly higher SOD activity in relation to the spermatozoa with high motility. The results confirmed the already known fact that SOD has an important role in removal of O_2^- to H_2O_2. However, high activity of SOD in spermatozoa is an indicator of disturbances in the spermatogenesis, with cytoplasmic retention as a consequence (O'FLAHERTY, 2014). A high activity of SOD is also associated with impaired spermatozoa function, motility and morphology, and is positively correlated with indicators of lipid damage (AITKEN et al., 1996, O'FLAHERTY, 2014). In this study, the fraction of abnormal spermatozoa, that contains spermatozoa with cytoplasmic droplets, had significantly higher SOD activity, which is in accordance with previous findings. Similar results were obtained by AITKEN et al. (1996), who found that human spermatozoa with low motility, spermatozoa with cytoplasmic droplets and morphologically abnormal spermatozoa had significantly higher SOD activity and significantly higher concentrations of lipid peroxides as compared to the spermatozoa fraction with high motility.

In addition, AITKEN et al. (1996) reported that spermatozoa with high motility have a small amount of cytoplasm and low SOD activity, which is in agreement with the results obtained in this study. Significantly higher values of TAS and GSH-Px were obtained in spermatozoa with very low motility than in the spermatozoa with high motility. The obtained results indicate the enhanced antioxidant defence response in the spermatozoa fraction with very low motility, due to increased production of ROS in that spermatozoa fraction, as BEER-LJUBIĆ et al. (2012) reported similarly for the fraction of bull spermatozoa with very low motility. They found significantly higher concentrations of lipid peroxides in spermatozoa with very low motility than in the spermatozoa fractions with high motility. The results of significantly higher SOD activity in the spermatozoa with very low motility in relation to spermatozoa with high motility could be also interpreted in relation to the results obtained for lipid peroxides, as reported by BEER-LJUBIĆ et al. (2012). Also, it is well known that the SOD activity is positively correlated to the concentration of lipid peroxidesand negatively to spermatozoa motility (AITKEN et al., 1996). Significantly lower values of TAS and GSH-Px found in abnormal spermatozoa indicate a decrease in the antioxidative protection of such spermatozoa. The abnormal

spermatozoa fraction obtained contained morphologically abnormal spermatozoa, spermatozoa with cytoplasmic droplets, and detached spermatozoa heads and tails, known to produce excessive amount of ROS, which may lead to oxidative stress and infertility (DE LAMIRANDE and GAGNON, 1995; AITKEN and SAWYER, 2003).

Conclusions

Higher values of SOD obtained in abnormal spermatozoa and spermatozoa with very low motility were associated with impaired spermatozoa function, motility and morphology. The significantly lower values of TAS and GSH-Px found in abnormal spermatozoa indicated a decrease in their antioxidative protection. The significantly higher levels of TAS and GSH-Px found in boar spermatozoa with low motility than in the spermatozoa with high motility could indicate enhanced antioxidative protection due to the increased production of ROS in the spermatozoa fraction with low motility. The spermatozoa with high motility and normal morphology could be applied in assisted reproduction and embryo transfer, but an intensive study of other antioxidant parameters, as well as evaluation of their values in the above mentioned spermatozoa fractions of boars may contribute to a better understanding of fertilizing properties and causes of infertility. Hence, in the future research it would be appropriate to determine the additional parameters of oxidative stress in boars.

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SAŽETAK

Negativni učinci prekomjerno stvorenih reaktivnih kisikovih spojeva (ROS) u sjemenu nerasta neutraliziraju se antioksidacijskim obrambenim mehanizmima. Budući da oplodnja i pohrana sjemena ovise o udjelu subpopulacija spermija (frakcija) u sjemenu cilj je ovog istraživanja bio odrediti pokazatelje antioksidacijskoga sustava u frakcijama spermija nerasta odvojenih u otopini jodiksanola. Uzorci sjemena 27 rasplodnih nerasta u dobi od 1,5 do 3 godine dobiveni su ručnom fiksacijom penisa, jednokratno u ranu jesen. Uzorci su bili centrifugirani u otopini jodiksanola diskontinuiranog gradijenta gustoće, a dobivene su tri frakcije spermija: velike gibljivosti, vrlo male gibljivosti i patološki oblici. U dobivenim frakcijama spermija određivane su vrijednosti ukupnog antioksidacijskog statusa (engl. pokrata TAS), superoksid dismutaze (engl. pokrata SOD) i glutation peroksidaze (engl. pokrata GSH-Px) pomoću spektrofotometra. Vrijednosti TAS-a i GSH-Px-a bile su značajno manje ($P<0,001$), a SOD-a značajno veća u patoloških oblika spermija u odnosu na spermije velike ili vrlo male gibljivosti ($P<0,001$, odnosno $P<0,05$). U spermijima vrlo male gibljivosti utvrđene su značajno veće vrijednosti TAS-a, SOD-a i GSH-Px-a u odnosu na spermije velike gibljivosti ($P<0,001$). Značajno veće vrijednosti SOD-a, dobivene u abnormalnim spermijima i u spermijima vrlo male gibljivosti, povezane su s poremećenom funkcijom, morfologijom i gibljivošću spermija. Najmanje vrijednosti TAS-a i GSH-Px-a utvrđene u patoloških oblika spermija upućuju na smanjenu antioksidacijsku zaštitu. Značajno veće vrijednosti TAS-a i GSH-Px-a utvrđene u spermijima vrlo male gibljivosti u odnosu na spermije velike gibljivosti ($P<0,001$) mogle bi upućivati na pojačan antioksidacijski obrambeni odgovor zbog pojačanog stvaranja ROS-a. Može se zaključiti da podatci o antioksidacijskim pokazateljima i njihova usporedba među dobivenim frakcijama spermija mogu pomoći u pojašnjenju uzroka neplodnosti nerasta.

Ključne riječi: antioksidansi, jodiksanol, spermiji, nerast